# EXHIBIT R

## TO DECLARATION OF SCOTT D. TANNER, PHD.

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# Metallobiomolecules. The basis of life, the challenge of atomic spectroscopy

Norbert Jakubowski,[a] Ryszard Lobinski[b] and Luc Moens[c]

[a]*Institute of Spectrochemistry and Applied Spectroscopy (ISAS), P. O. Box 10 13 52, D-44013 Dortmund, Germany. E-mail: jakubowski@isas-dortmund.de*
[b]*Equipe de Chimie Analytique Bio-inorganique, CNRS UMR 5034, Helioparc, 2 av. Pierre Angot, 64053 Pau, France and Department of Analytical Chemistry, Warsaw University of Technology, u. Noakowskiego 3, 00-664 Warsaw, Poland. E-mail: ryszard.lobinski@univ-pau.fr*
[c]*Laboratory of Analytical Chemistry, Ghent University, Proeftuinstraat 86, B-9000 Ghent, Belgium. E-mail: luc.moens@rug.ac.be*

An outline of recent advances in metalloproteomics (structural and functional characterization of metal-binding proteins and their structural metal-binding moieties) and metallomics (characterization of the entirety of metal and metalloid species within a cell or tissue type) is presented. ICP-MS allows the specific detection of heteroelements after separation using techniques traditionally associated with biochemistry, such as gel electrophoresis or capillary chromatography. The element specificity, independence of matrix effects, and wide linear range make ICP-MS an attractive complementary technique to ESI/MALDI-MS for the analysis of sulfur-, selenium- and phosphorus-containing proteins, metal-complexes with nucleic acids, carbohydrates and proteins, and metal-tagged biomolecules in general.

## Introduction

Most elements play a role in life, and all elements in the periodic table can be detected by atomic spectroscopy. Atomic spectroscopy has for a long time contributed to life sciences by answering the question: which elements at what concentration play an important role in life processes? As the sensitivity of atomic spectroscopy and the level of contamination control has improved an increasing number of elements have been found to be essential. But even now many processes involving trace elements are far from well understood because most matrices of biological interest, e.g., body fluids, tissues or foods, are very complex and hence difficult to analyse. Studies of bioavailability can be difficult, hampered by the synergistic, competing or complementing effects of many elements.

There are also elements (usually heavy metals) that have been identified to play a negative role in living systems by being toxic, mutagenic or carcinogenic. The increasing number of technological processes and products used in modern life results in our coming into contact with more compounds at higher concentrations than ever before. An increasing number of elements are released into the environment and enter the food chain, while the biological effects of this exposure often remain virtually unknown.

In the past most analytical problems relating to biological systems were addressed by measuring the total concentrations of elements. Now there is increasing awareness of the importance of the chemical form in which an element is present in biological systems, e.g., the oxidation state, the nature of the ligands or even the molecular structure. Information on speciation is critically required in order to understand biological processes, and atomic spectroscopy is fast becoming one of the most important tools for such speciation studies. The definition, philosophy, methods and novel aspects of speciation have recently been addressed elsewhere.[1,2]

The availability of the complete sequence of several genomes offers unprecedented opportunities to identify all metalloproteins and their enzymatic metabolites (see Maret's review in this

issue, p. 15). It allows a comprehensive look at the role of essential and toxic metals in health and disease and has given considerable momentum to the fields of metalloproteomics (structural and functional characterization of metal-binding proteins and their structural metal-binding moieties) and metallomics (characterization of the entirety of metal and metalloid species within a cell or tissue type, see the review by Haraguchi, p. 5 in this issue). The tagging of biomolecules with a metal or a metal compound in order to make the biomolecule "visible" by a sensitive element-specific detector is emerging as an important area of research. Elemental tags and their detection by atomic spectroscopy are paving the way to biochemical and biotechnological analysis.[3]

The aim of this editorial and of this special issue is to demonstrate why, where, and how atomic spectroscopy can play a new role in the life sciences. Speciation in the life sciences has always been located at the interface between atomic and molecular spectroscopy—techniques that should be viewed as complementary and not competing.[4] This is the focus of this special issue. The borderline between traditional inorganic (ICP) and organic (electrospray and MALDI) mass spectrometry is becoming less well defined with ICP-MS entering the field of proteomics and electrospray MS becoming a key tool in speciation analysis. Applications in the life sciences are based on multi-method analytical approaches, where atomic spectroscopy plays an important role: many techniques are needed to understand the complex processes of life.

## Why atomic spectroscopy?

To date, analytical atomic spectrometry has not been tremendously popular for speciation of metallobiomolecules. Indeed, as pointed out in a comprehensive review by Szpunar,[5] an overwhelming majority of applications have been driven by the analytical chemistry community and not by biochemists. The latter have tended to prefer molecular spectroscopic techniques, such as circular dichroism spectroscopy (CDS) (see

required. The hyphenation of these separation techniques to atomic spectroscopy was delayed due to the fact that adequate sample introduction techniques had not been available. Since the pioneering work of Olesik et al.[10] CE has been coupled to ICP-MS instruments and a commercial interface is available.[11] It convinces by its high separation power, for instance for Se speciation in urine (see Gammelgaard and Bendahl, p. 135), or for Al speciation in cell cultures (see the communication of Polec-Pawlak et al. p. 41 in this issue). In a selenized yeast sample Bendahl and Gammelgaard achieved 620 000 theoretical plates (see p. 143).

One of the most important working tools for the separation of proteins is PAGE related separation techniques. Usually the protein spots are excised, tryptically digested, and the resulting peptides are analysed by MALDI-TOF-MS or ESI-MS (see the paper of Chassaigne et al. in this issue on p. 85) to identify a specific heteroelement or a (semi-)metal in the protein. An alternative approach was first described in the pioneering papers of McLeod and co-workers,[12] who used laser ablation of PAGE gels to identify directly proteins containing the metal of interest. PAGE-LA-ICP-MS has now become a fast growing application area for the analysis of biomolecules as demonstrated by a number of very recently published papers[13-16] (also see Becker et al., p. 149).

These examples demonstrate how atomic spectroscopy is discovering larger molecules and how spectroscopists are now confronting pure biochemical problems.

### Future instrumentation

In biochemistry, separation systems optimised for using small amounts and small volumes of samples (e.g., using micro- and nanobore columns) are currently used routinely. It can still be a challenge to couple atomic detectors with micro- and nanobore columns, usually applied in ESI-MS with flow rates of μl min$^{-1}$. Ultra-low flow nebulisers for atomic spectroscopy are still not widely available, but are necessary if atomic detectors are to be operated in parallel to molecular spectrometers (see review by Wind and Lehmann, p. 20, Montes-Bayon et al., p. 153 and also a paper by Adams and co-workers, p. 159, all in this issue). Hence the significance of coupling CE or capillary HPLC using minimum sheath flow[11] or sheathless interfaces, respectively, is a prerequisite.[17]

Atomic spectroscopy could gain popularity in biochemistry and biomedicine, if the instrumentation was smaller and easier to operate. For many applications, instruments are needed as detectors in LC applications only, and most often single elements are investigated. Is ICP-MS, an expensive multi-element method, the only choice here? There is still room for innovation—we are still far away from the "lab on the chip" approach we are waiting for. Plasmas will become miniaturised,[18] flame AAS will be equipped with laser diodes[19] or AAS even will become a multielement technique[20] and methods and procedures will be extensively elaborated for atomic fluorescence spectroscopy (see Aravind and Prasad p. 52, and for instance Dumont et al. p. 167) to make it more robust.

Another trend involves the development of instruments that allow elemental and molecular information to be obtained at the same time. As shown by Hieftje and co-workers,[21] two sources, an electrospray and an ICP-source, can be coupled to a single time-of-flight mass spectrometer by splitting the flows to the ionisation sources. For the moment this remains speculative, but it seems timely to develop hybrid instrumentation, multiple source instruments and arrangements consisting of multiple spectrometers operated in series and/or in parallel.[22]

Often used for the detection of metals and semimetals, ICP-MS has rarely been applied for the determination of the stoichiometry of biomolecules unless they contain a heteroelement. Indeed, the detection of C, N, O and H has been hampered by high blanks. For the detection of these elements

better sources, so-called tunable sources, are at hand, as discussed elsewhere.[23,24] A more recent example of how to measure both the atomic composition and the molecular structure of a molecule was given by Majidi and co-workers.[25] These examples clearly show the paradigm shift in spectroscopic research. Sources originally designed for atomic spectroscopy conquer molecular spectroscopy and vice versa.

Instrumentation in atomic spectroscopy is becoming more powerful. Detection limits are improving, reaching pico- or even femtomole levels in complex matrices. This now allows analysis of very small sample volumes. The analysis of single cells is a future challenge for spectroscopy, but for this purpose novel instrumentation is needed with new features. Instruments with higher spatial resolution are needed to look into cells and instruments with a higher temporal resolution to study the chemistry in real time, because the cell is not a static but a dynamic system. Metallomics of the cell should also be put in the context of genomics and proteomics.

### Future trends and challenges

Any biomolecule can be rendered detectable by atomic spectrometry by being tagged by a metal or its compound. The concept of elemental tags is commonly applied in biochemistry, including labelling with radioactive isotopes, neutron activation (see review by Chai et al., p. 26) or silver staining of polyacrylamide gels. With respect to radioactive labelling, ICP-MS is becoming an important competitor: using stable isotopes the same biomolecules can be detected with nearly the same sensitivity (see for instance Pergantis et al., p. 178); also, using stable, naturally occurring isotopes, radioactive stress of the biological system can be avoided and radioactive waste can be reduced drastically. Whereas previously special laboratory requirements and regulation have restricted wide-adoption of these techniques, now these applications are open for everyone. Elemental tags, especially rare earth elements, have been applied for combinatorial chemistry.[26] A phosphate capture molecule (zinc-isotope derivative) has been employed for the analysis of phosphorylated compounds.[27] Methods for sequencing argentinated peptides by electrospray[28] and MALDI[29] have been proposed but not HPLC-ICP-MS applications. Pioneering work addressing the development of a sensitive and quantitative element-tagged immunoassay with ICP-MS detection has been reported by Baranov et al.[30] The use of stable isotope labelled tags is a possible route to achieve quantitative protein analysis but unfortunately one of the key elements, phosphorus, is monoisotopic. The precision and accuracy of quadrupole analysers or even emission spectroscopy (Schmidt et al., p.172) appear for the moment sufficient for isotope dilution quantifications but studies of the natural fractionation will require the use of ICP-multicollector instruments: for instance, it has been shown by Walczyk and von Blanckenburg that even elements such as iron show a natural fractionation which is different depending on sex.[31]

Elemental detectors have reached a very high level of utility in biochemistry, nutrition and medicine but there are still many problems which remain unsolved. Many techniques applied routinely in biochemistry cannot be directly used with elemental detection. The reason for this is the ubiquitous presence of many elements of interest in the buffers used during separation or the risk of contamination. An obvious example is that use of phosphate buffers (usually applied for SDS-PAGE) makes the detection of phosphorus in biomolecules difficult. Sulfur cannot be used as an elemental tag for a protein in SDS-PAGE separations due to extremely high blanks. Here new separation techniques and procedures have to be developed to explore the capabilities of elemental detection.

Many metal complexes with biomolecules are relatively

Keppler and co-workers, p. 46 of this issue), electron paramagnetic resonance (EPR) (see Gutschank et al., p. 34), extended X-ray absorption fine structure/near edge structure (EXAFS/XANES), NMR and, more recently, mass spectrometry and even traditional atomic spectroscopy are often applied. The latter holds true for flame AAS and graphite furnace AAS (see Aravind and Prasad, p. 52, and Van Hulle et al., p. 58) or atomic emission spectrometry (see Uden et al., p. 65). The limitations of the above techniques become more evident the lower the concentrations of metals involved and the more complex the analytical sample. Very sensitive and specific immunoassays can be mentioned here as an exception.

The indisputable advantage of ICP-MS is its capability for discriminating (with isotopic resolution) between metal (heteroelement)-containing species and metal-free species in a HPLC or capillary electrophoresis effluent or, via laser ablation, in a 2-D gel spot. Recent key developments in ICP-MS of potential significance for metallomics were discussed by Szpunar in a recent paper:[6]

(i) the advent of collision/reaction cell instruments and sector field devices (see Zheng and Hintelmann, p. 191 and Koellensperger et al., p. 74), extending the measurable elements to phosphorus and sulfur (see Goessler and co-workers, p. 80) and improving the isotope ratio measurements of essential elements such as selenium, chromium, vanadium and iron;

(ii) the development of interfaces allowing the introduction of effluents from capillary (id 300 µm) and soon from nanocapillary (id <150 µm) columns via a direct injection or a total consumption nebuliser resulting in sub-femtogram detection limits in dry plasma conditions; and

(iii) scanning 2-D gels for the presence of metal-containing spots by laser ablation ICP-MS, offering a cheaper alternative to the existing synchrotron radiation XRF techniques used for this purpose (see Chassaigne et al., p. 85).

Bearing in mind these developments, atomic spectroscopy is not only capable of measuring the total element concentration in a biological sample, but is also capable of measuring the stoichiometry of the heteroelements in a biomolecule isolated as a spot in 2-D gel electrophoresis or in a tiny amount of a narrow capillary electrophoretic peak.[7]

When employed as a detector in size-exclusion LC, ICP-MS allows the estimation of the molecular weight of biomolecules in non-denaturing conditions. Novel applications of ICP-MS for biochemical and biomedical research are reviewed in this issue by Wind and Lehmann (see p. 20). Among others, medical applications, for instance applications in cancer research (see Bandura et al., p. 96), dialysis (Kralj et al. p. 101), cardiovascular tissues and quality control of pharmaceuticals (see Caruso and co-workers, p. 107) are emerging as hot topics here.

The limits of ICP-MS are at present defined by its inability to elucidate the structure or identify the metallobiomolecules for which retention/migration time standards are not available. It is here that molecular mass spectrometry, combined with different ionization techniques such as ESI[4] and matrix assisted laser desorption ionisation (MALDI),[8] has a clear role to play (see Szpunar and co-workers, p. 114).

These techniques allow the determination of the exact molecular weight by which the molecule can be identified. In ideal conditions ESI and MALDI have outstanding sensitivity so that biomolecules can be analysed at femtomole levels. However, the biomolecule needs to be charged, and matrix suppression effects in electrospray MS cannot be underestimated. Zwitterions, such as, for example, some selenium species are difficult to ionise and therefore show worse limits of detection, a reason why ICP-MS is so often applied for this purpose (see a recent review by Uden et al.).[9] Slightly different compounds might show significant variations in sensitivity, thus calibration is needed, but standards are often not available and therefore have to be synthesized. Here atomic spectrometry

(in particular, ICP-MS) is a complementary method. Sensitivity is proportional to the number of atoms in the biomolecule and atoms can be ionized in the plasma. Calibration can often be performed using liquid standards and, due to the capability of a substance independent calibration, even inorganic compounds can be used for this purpose.

## Element specific detection of metallobiomolecules

The specific detection of a metal compound in a cell line, plant, animal or human tissue needs to be achieved first before any further studies on its identification and characterisation. The actual problem can be simplified provided that the analytical signal detected by an instrument is due to the presence of the metal compound, and not to that of a simple ion, or a ligand.[6]

Detection can be achieved by hyphenated techniques that combine micro-scale isolation and purification and a sensitive, element specific detector, usually ICP-MS. However, other detectors can also be used. Most inorganic analyses in biochemistry or medical laboratories still utilise traditional methods, such as flame and graphite furnace AAS (see Aravind and Prasad p. 52 and Van Hulle et al. p. 58) or atomic emission spectrometry (see Uden et al. p. 65). Sensitivity is, however, becoming a key issue when capillary separation techniques are used or when the target sample is small (e.g., individual cells).

Fig. 1 details the different isolation and purification techniques applied for biomolecules of various sizes. On the x-axis the capacity of the various separation techniques is given, expressed as the number of substances which can be separated. On the y-axis the operational region in terms of molecular weight is given. For smaller molecules and less complex samples, HPLC techniques, including ion chromatography and reversed-phase chromatography, look especially promising in terms of their separation capacity. Applications relating to small molecules are still the domain for atomic spectroscopy. For more complex samples multidimensional chromatographic systems (see for instance Michalke and Schramel, p. 121 and Bouyssiere et al., p. 196) prove to be more effective, although atomic spectroscopy is still often used as the detector.

As is shown in Fig. 1, for the separation of larger molecules and more complex samples, the application of size exclusion chromatography (SEC) (see Daun et al., p. 129), electrophoresis, capillary electrophoresis (CE), capillary electrochromatography (CEC) or polyacrylamide gel electrophoresis (PAGE) is
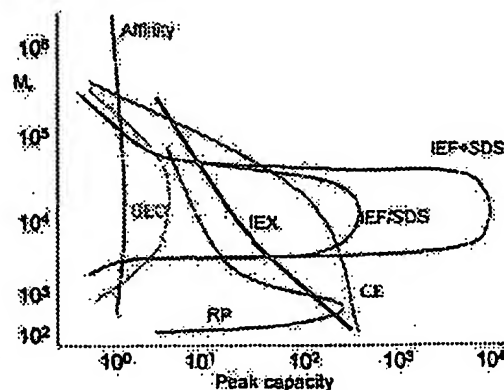


Fig. 1  Isolation and purification techniques applied for biomolecules of different sizes. Key: affinity = affinity chromatography; CE = capillary electrophoresis; IEF = isoelectric focusing; IEX = ion exchange chromatography; SDS = sodium dodecyl sulfate PAGE; SEC = size exclusion chromatography; RP = reversed phase chromatography. Reproduced with permission of F. Lõttspeich, Max Planck Institute for Biochemistry, Martinsried, Germany.

weak. Chromatography of picogram or nanogram quantities of metallocompounds has not reached maturity yet and many phenomena, such as metal absorption or ligand exchange, at these levels are poorly understood and difficult to investigate. Many (semi-)metal complexes or proteins are not stable and can easily adhere to the wall of an electrophoretic capillary or stationary phase in chromatography (see, for instance, the paper by Feldmann and co-workers in this issue, p. 183). Here again new separation techniques, procedures and strategies are urgently needed.

Another problem is the quality control in metallomics analysis. Retention or migration time standards are usually unavailable and need to be custom synthesized to confirm identification and to allow quantitation. Certified reference materials for speciation analysis are available only for covalent contaminants (e.g. butyltin or methylmercury) (see http://www-nuweb.iaea.org/nahu/external/e4/nmrm/; http://www.element-speciation.net) and have a limited value for metallomics. For metallobiomolecules new standard materials are urgently required, not only for identification, but also for traceability and comparability of approaches developed for the life sciences. It is becoming increasingly difficult to assess the different methods developed and applied so far for this purpose as long as a validation of results is not given by independent methods or the analysis of reference materials.

Metallomic data should not be considered separately from the genome and the proteome of a cell. This is somewhat analogous to combining *in vivo* bioanalytical data with *in vitro* molecular genetic data, as demonstrated recently for the identification of the Ni–nicotianamine complex in plants.[32] The wider use of molecular biological methods is expected to complement the analytical metallomics and lead to understanding of metalloprotein functions at the molecular level.

## Summary

This special issue of *JAAS* is a collection of papers demonstrating applications of analytical and atomic spectroscopy in the life sciences. We believe that this area will continue to be a hot topic for this journal and we strongly encourage those authors working in this field to contribute their research articles to *JAAS*.

We, the guest editors, still have a dream—slowly but continuously the revolution in life sciences is taking place and analytical methods are strongly involved. We are reaching the point in the life sciences where joint efforts are needed to start solving the problems of tomorrow today and every one of us (the readers) can contribute.

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[a]Institute of Spectrochemistry and Applied Spectroscopy (ISAS), P. O. Box 10 13 52, D-44013 Dortmund, Germany. E-mail: jakubowski@isas-dortmund.de
[b]Equipe de Chimie Analytique Bio-inorganique, CNRS UMR 5034, Helioparc, 2 av. Pierre Angot, 64053 Pau, France and Department of Analytical Chemistry, Warsaw University of Technology, u. Noakowskiego 3, 00-664 Warsaw, Poland. E-mail: ryszard.lobinski@univ-pau.fr
[c]Laboratory of Analytical Chemistry, Ghent University, Proeftuinstraat 86, B-9000 Ghent, Belgium. E-mail: luc.moens@rug.ac.be

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### Future instrumentation

In biochemistry, separation systems optimised for using small amounts and small volumes of samples (e.g., using micro- and nanobore columns) are currently used routinely. It can still be a challenge to couple atomic detectors with micro- and nanobore columns, usually applied in ESI-MS with flow rates of $\mu$l min$^{-1}$. Ultra-low flow nebulisers for atomic spectroscopy are still not widely available, but are necessary if atomic detectors are to be operated in parallel to molecular spectrometers (see review by Wind and Lehmann, p. 20, Montes-Bayon et al., p. 153 and also a paper by Adams and co-workers, p. 159, all in this issue). Hence the significance of coupling CE or capillary HPLC using minimum sheath flow[11] or sheathless interfaces, respectively, is a prerequisite.[17]

Atomic spectroscopy could gain popularity in biochemistry and biomedicine, if the instrumentation was smaller and easier to operate. For many applications, instruments are needed as detectors in LC applications only, and most often single elements are investigated. Is ICP-MS, an expensive multi-element method, the only choice here? There is still room for innovation—we are still far away from the "lab on the chip" approach we are waiting for. Plasmas will become miniaturised,[18] flame AAS will be equipped with laser diodes[19] or AAS even will become a multielement technique[20] and methods and procedures will be extensively elaborated for atomic fluorescence spectroscopy (see Aravind and Prasad p. 52, and for instance Dumont et al. p. 167) to make it more robust.

Another trend involves the development of instruments that allow elemental and molecular information to be obtained at the same time. As shown by Hieftje and co-workers,[21] two sources, an electrospray and an ICP-source, can be coupled to a single time-of-flight mass spectrometer by splitting the flows to the ionisation sources. For the moment this remains speculative, but it seems timely to develop hybrid instrumentation, multiple source instruments and arrangements consisting of multiple spectrometers operated in series and/or in parallel.[22]

Often used for the detection of metals and semimetals, ICP-MS has rarely been applied for the determination of the stoichiometry of biomolecules unless they contain a heteroelement. Indeed, the detection of C, N, O and H has been hampered by high blanks. For the detection of these elements

better sources, so-called tunable sources, are at hand, as discussed elsewhere.[23,24] A more recent example of how to measure both the atomic composition and the molecular structure of a molecule was given by Majidi and co-workers.[25] These examples clearly show the paradigm shift in spectroscopic research. Sources originally designed for atomic spectroscopy conquer molecular spectroscopy and vice versa.

Instrumentation in atomic spectroscopy is becoming more powerful. Detection limits are improving, reaching pico- or even femtomole levels in complex matrices. This now allows analysis of very small sample volumes. The analysis of single cells is a future challenge for spectroscopy, but for this purpose novel instrumentation is needed with new features. Instruments with higher spatial resolution are needed to look into cells and instruments with a higher temporal resolution to study the chemistry in real time, because the cell is not a static but a dynamic system. Metallomics of the cell should also be put in the context of genomics and proteomics.

### Future trends and challenges

Any biomolecule can be rendered detectable by atomic spectrometry by being tagged by a metal or its compound. The concept of elemental tags is commonly applied in biochemistry, including labelling with radioactive isotopes, neutron activation (see review by Chai et al., p. 26) or silver staining of polyacrylamide gels. With respect to radioactive labelling, ICP-MS is becoming an important competitor: using stable isotopes the same biomolecules can be detected with nearly the same sensitivity (see for instance Pergantis et al., p. 178); also, using stable, naturally occurring isotopes, radioactive stress of the biological system can be avoided and radioactive waste can be reduced drastically. Whereas previously special laboratory requirements and regulation have restricted wide-adoption of these techniques, now these applications are open for everyone. Elemental tags, especially rare earth elements, been applied for combinatorial chemistry.[26] A phosphate capture molecule (zinc-isotope derivative) has been employed for the analysis of phosphorylated compounds.[27] Methods for sequencing argentinated peptides by electrospray[28] and MALDI[29] have been proposed but not HPLC-ICP-MS applications. Pioneering work addressing the development of a sensitive and quantitative element-tagged immunoassay with ICP-MS detection has been reported by Baranov et al.[30] The use of stable isotope labelled tags is a possible route to achieve quantitative protein analysis but unfortunately one of the key elements, phosphorus, is monoisotopic. The precision and accuracy of quadrupole analysers or even emission spectroscopy (Schmidt et al., p.172) appear for the moment sufficient for isotope dilution quantifications but studies of the natural fractionation will require the use of ICP-multicollector instruments: for instance, it has been shown by Walczyk and von Blanckenburg that even elements such as iron show a natural fractionation which is different depending on sex.[31]

Elemental detectors have reached a very high level of utility in biochemistry, nutrition and medicine but there are still many problems which remain unsolved. Many techniques applied routinely in biochemistry cannot be directly used with elemental detection. The reason for this is the ubiquitous presence of many elements of interest in the buffers used during separation or the risk of contamination. An obvious example is that use of phosphate buffers (usually applied for SDS-PAGE) makes the detection of phosphorus in biomolecules difficult. Sulfur cannot be used as an elemental tag for a protein in SDS-PAGE separations due to extremely high blanks. Here new separation techniques and procedures have to be developed to explore the capabilities of elemental detection.

Many metal complexes with biomolecules are relatively

Keppler and co-workers, p. 46 of this issue), electron paramagnetic resonance (EPR) (see Gutschank et al., p. 34), extended X-ray absorption fine structure/near edge structure (EXAFS/XANES), NMR and, more recently, mass spectrometry and even traditional atomic spectroscopy are often applied. The latter holds true for flame AAS and graphite furnace AAS (see Aravind and Prasad, p. 52, and Van Hulle et al., p. 58) or atomic emission spectrometry (see Uden et al., p. 65). The limitations of the above techniques become more evident the lower the concentrations of metals involved and the more complex the analytical sample. Very sensitive and specific immunoassays can be mentioned here as an exception.

The indisputable advantage of ICP-MS is its capability for discriminating (with isotopic resolution) between metal (heteroelement)-containing species and metal-free species in a HPLC or capillary electrophoresis effluent or, via laser ablation, in a 2-D gel spot. Recent key developments in ICP-MS of potential significance for metallomics were discussed by Szpunar in a recent paper:[6]

(i) the advent of collision/reaction cell instruments and sector field devices (see Zheng and Hintelmann, p. 191 and Koellensperger et al., p. 74), extending the measurable elements to phosphorus and sulfur (see Goessler and co-workers, p. 80) and improving the isotope ratio measurements of essential elements such as selenium, chromium, vanadium and iron;

(ii) the development of interfaces allowing the introduction of effluents from capillary (id 300 µm) and soon from nanocapillary (id <150 µm) columns via a direct injection or a total consumption nebuliser resulting in sub-femtogram detection limits in dry plasma conditions; and

(iii) scanning 2-D gels for the presence of metal-containing spots by laser ablation ICP-MS, offering a cheaper alternative to the existing synchrotron radiation XRF techniques used for this purpose (see Chassaigne et al., p. 85).

Bearing in mind these developments, atomic spectroscopy is not only capable of measuring the total element concentration in a biological sample, but is also capable of measuring the stoichiometry of the heteroelements in a biomolecule isolated as a spot in 2-D gel electrophoresis or in a tiny amount of a narrow capillary electrophoretic peak.[7]

When employed as a detector in size-exclusion LC, ICP-MS allows the estimation of the molecular weight of biomolecules in non-denaturing conditions. Novel applications of ICP-MS for biochemical and biomedical research are reviewed in this issue by Wind and Lehmann (see p. 20). Among others, medical applications, for instance applications in cancer research (see Bandura et al., p. 96), dialysis (Kralj et al. p. 101), cardiovascular tissues and quality control of pharmaceuticals (see Caruso and co-workers, p. 107) are emerging as hot topics here.

The limits of ICP-MS are at present defined by its inability to elucidate the structure or identify the metallobiomolecules for which retention/migration time standards are not available. It is here that molecular mass spectrometry, combined with different ionization techniques such as ESI[4] and matrix assisted laser desorption ionisation (MALDI),[8] has a clear role to play (see Szpunar and co-workers, p. 114).

These techniques allow the determination of the exact molecular weight by which the molecule can be identified. In ideal conditions ESI and MALDI have outstanding sensitivity so that biomolecules can be analysed at femtomole levels. However, the biomolecule needs to be charged, and matrix suppression effects in electrospray MS cannot be underestimated. Zwitterions, such as, for example, some selenium species are difficult to ionise and therefore show worse limits of detection, a reason why ICP-MS is so often applied for this purpose (see a recent review by Uden et al.).[9] Slightly different compounds might show significant variations in sensitivity, thus calibration is needed, but standards are often not available and therefore have to be synthesized. Here atomic spectrometry

(in particular, ICP-MS) is a complementary method. Sensitivity is proportional to the number of atoms in the biomolecule and atoms can be ionized in the plasma. Calibration can often be performed using liquid standards and, due to the capability of a substance independent calibration, even inorganic compounds can be used for this purpose.

### Element specific detection of metallobiomolecules

The specific detection of a metal compound in a cell line, plant, animal or human tissue needs to be achieved first before any further studies on its identification and characterisation. The actual problem can be simplified provided that the analytical signal detected by an instrument is due to the presence of the metal compound, and not to that of a simple ion, or a ligand.[6]

Detection can be achieved by hyphenated techniques that combine micro-scale isolation and purification and a sensitive, element specific detector, usually ICP-MS. However, other detectors can also be used. Most inorganic analyses in biochemistry or medical laboratories still utilise traditional methods, such as flame and graphite furnace AAS (see Aravind and Prasad p. 52 and Van Hulle et al. p. 58) or atomic emission spectrometry (see Uden et al. p. 65). Sensitivity is, however, becoming a key issue when capillary separation techniques are used or when the target sample is small (e.g., individual cells).

Fig. 1 details the different isolation and purification techniques applied for biomolecules of various sizes. On the x-axis the capacity of the various separation techniques is given, expressed as the number of substances which can be separated. On the y-axis the operational region in terms of molecular weight is given. For smaller molecules and less complex samples, HPLC techniques, including ion chromatography and reversed-phase chromatography, look especially promising in terms of their separation capacity. Applications relating to small molecules are still the domain for atomic spectroscopy. For more complex samples multidimensional chromatographic systems (see for instance Michalke and Schramel, p. 121 and Bouyssiere et al., p. 196) prove to be more effective, although atomic spectroscopy is still often used as the detector.

As is shown in Fig. 1, for the separation of larger molecules and more complex samples, the application of size exclusion chromatography (SEC) (see Daun et al., p. 129), electrophoresis, capillary electrophoresis (CE), capillary electrochromatography (CEC) or polyacrylamide gel electrophoresis (PAGE) is
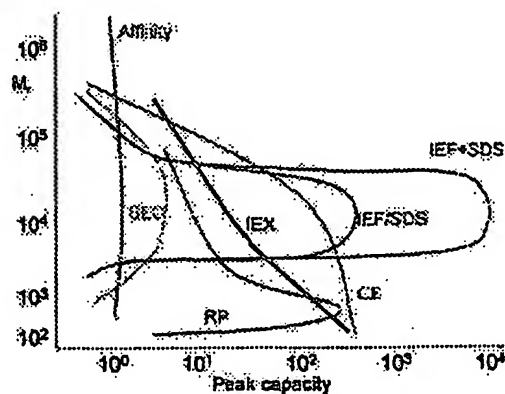


Fig. 1 Isolation and purification techniques applied for biomolecules of different sizes. Key: affinity = affinity chromatography; CE = capillary electrophoresis; IEF = isoelectric focusing; IEX = ion exchange chromatography; SDS = sodium dodecyl sulfate PAGE; SEC = size exclusion chromatography; RP = reversed phase chromatography. Reproduced with permission of F. Löttspeich, Max Planck Institute for Biochemistry, Martinsried, Germany.

weak. Chromatography of picogram or nanogram quantities of metallocompounds has not reached maturity yet and many phenomena, such as metal absorption or ligand exchange, at these levels are poorly understood and difficult to investigate. Many (semi-)metal complexes or proteins are not stable and can easily adhere to the wall of an electrophoretic capillary or stationary phase in chromatography (see, for instance, the paper by Feldmann and co-workers in this issue, p. 183). Here again new separation techniques, procedures and strategies are urgently needed.

Another problem is the quality control in metallomics analysis. Retention or migration time standards are usually unavailable and need to be custom synthesized to confirm identification and to allow quantitation. Certified reference materials for speciation analysis are available only for covalent contaminants (*e.g.* butyltin or methylmercury) (see http://www-naweb.iaea.org/nahu/external/e4/nmrm/; http://www.element-speciation.net) and have a limited value for metallomics. For metallobiomolecules new standard materials are urgently required, not only for identification, but also for traceability and comparability of approaches developed for the life sciences. It is becoming increasingly difficult to assess the different methods developed and applied so far for this purpose as long as a validation of results is not given by independent methods or the analysis of reference materials.

Metallomic data should not be considered separately from the genome and the proteome of a cell. This is somewhat analogous to combining *in vivo* bioanalytical data with *in vitro* molecular genetic data, as demonstrated recently for the identification of the Ni–nicotianamine complex in plants.[32] The wider use of molecular biological methods is expected to complement the analytical metallomics and lead to understanding of metalloprotein functions at the molecular level.

## Summary

This special issue of *JAAS* is a collection of papers demonstrating applications of analytical and atomic spectroscopy in the life sciences. We believe that this area will continue to be a hot topic for this journal and we strongly encourage those authors working in this field to contribute their research articles to *JAAS*.

We, the guest editors, still have a dream—slowly but continuously the revolution in life sciences is taking place and analytical methods are strongly involved. We are reaching the point in the life sciences where joint efforts are needed to start solving the problems of tomorrow today and every one of us (the readers) can contribute.

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